Review

Cross-Sectional Use of Vascular, Blood-Based Biomarkers—Augmenting Postmortem

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Abstract: Many assets of clinical medicine, such as clinical chemistry and diagnostic imaging, make clinical diagnosis outstanding compared to postmortem diagnosis. An assessment of functional status certainly has priority over the postmortem, cross-sectional use of diagnostic tests and laboratory equipment. In addition, the cost of these tools is sometimes steep, and their use does not always fit into a reasonable cost–benefit ratio. However, sometimes postmortem observations, such as inflammation, pulmonary edema, or infiltration and cerebral swelling, cannot be explained without implementing immunohistochemical markers for postmortem diagnosis. Introducing blood-based biomarkers in postmortem care could significantly reduce the rates of inconclusive postmortems and discrepancies in autopsy findings and clinical diagnoses. This is particularly relevant in the scope of vascular pathology, considering the great burden of vascular diseases in overall mortality. Expanding traditional autopsy with blood-based (circulating) biomarkers to avoid invasive postmortem examination would have cultural, religious, and potential economic advantages. All of the target molecules are discussed in the context of the process they up-regulate or down-regulate and which turns out to be the final cause of death. Ultimately, it is evident that further studies are needed to provide concrete validation for a combination of markers on a specific case order to reach a postmortem diagnosis with or without clinical records.

Keywords: blood-based biomarkers; clotting; inflammation; postmortem; vascular biomarkers

1. Introduction

All that we know about normal circulation was obtained from exhaustive postmortem dissection [1]. More than 40 years ago, in a series of 500 clinical autopsies, vascular disorders were found to account for 25.2% of anatomopathological diagnoses [2]. These figures are much the same as those in osteoarthritis/rheumatoid arthritis research from 2015 [3]. Data from the Eurostat indicate the same effect: diseases of the circulatory system are the main cause of death in the EU, responsible for almost 37% of all deaths in 2017 [4,5]. Earle et al. recently presented data on the cause of death in patients with risk of pulmonary embolism (PE), and their figures were basically in correspondence [6]. Clinical decision rules in combination with a D-dimer assay were applied to exclude PE [7]. The lack of circulating oxygen, altered enzymatic reactions, cellular degradation, and cessation of anabolic production of metabolites all cause very extensive biochemical changes in all body tissues postmortem [8]. Aside from PE implications, the real potential of D-dimer as a biomarker was realized during the COVID-19 pandemic, where it was used to examine disease severity and mortality in patients in a case–control study [9].

Etymologically, the term "biomarker" comes from the Greek combining the form βιο-, from βίος ("life"), and Old English forms of words meaning a mark [10,11]. Therefore, bearing in mind this Greek root, using a word for life in the context of postmortem might seem a bit odd. However, a biomarker may be a recording taken from an individual; it may be an imaging test, or it may be a biosample.

Biomarkers found in body fluids may represent the active disease process or the patient's reaction to the disease [12]. Notwithstanding, their purpose is to act as an alternate outcome measure to assess the efficacy of therapy. The most common understanding is that of a biomarker as a protein, enzyme, or cytokine with discriminatory value in clinical care [13,14]. A heterogeneity of molecules has been evaluated, and although postmortem biomarkers and multimarker strategies are best investigated in the light of sudden cardiac death and agonal cardiac function [15,16], their great potential at the level of peripheral vasculature is yet to be examined [12,17]. Any biomarker, whatsoever, has to meet certain criteria to be a surrogate endpoint, or to be able to predict a clinically relevant endpoint, such as loss of

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vision or a decrease in quality of life. In addition, the effect of a proposed treatment on the surrogate must capture the effect of the treatment on the clinically relevant endpoint [18,19].

This should be considered in the context of the fact that autopsies are facing many challenges. Sometimes, governmental funding to hospital-based autopsies is not regulated, or hospitals reject autopsies requested by the family [20]. Either way, autopsy numbers have been dropping significantly [21–23], and postmortem healthcare is unequally accessible [24].

2. Postmortem Biomarkers

Biomarkers provide plenty of information to enhance all aspects of vascular homeostasis through vascular beds [12]. Biomarkers are characteristic indicators of disease, disease state, or disease progression. At first described as a "measurable and quantifiable biological parameter that could serve as an index for health assessment", they were ultimately differentiated into "a characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [25,26].

The postmortem period does include events such as autolysis or decay, and biomarkers found in body fluids may represent the active disease process or reaction to disease. Therefore, the value of postmortem biomarkers should be evaluated bearing that in mind, even if their efficacy is clinically confirmed [27]. This adds new value to clinical postmortem studies, not only as a method of control, but to improve teaching methods in hospitals [23].

The augmentation of postmortem examinations with blood-based (circulating) biomarkers in adults to avoid invasive autopsy would have cultural, religious, and potential economic benefits [28–30]. In fact, no contemporary studies have compared the actual costs of postmortem optional modalities.

3. Biomarkers of Vascular Homeostasis

Endothelial quiescence and normality are important for disease resistance. Circulating, blood-based biomarkers are simply pointers of organ-specific signaling pathways [31]. The vascular system has a resting layer of endothelial cells (EC) that do not divide. This layer of long-lived cells of the mesodermal lineage that lines the inside of all blood vessels forms a single layer of organotypically differentiated cells [32]. This is known as vascular quiescence, and scientists know little about how the body achieves and maintains it.

3.1. Vascular Biomarkers and Omics

Samples of body fluids and vascular tissue used in vascular biomarker discovery have been assessed using genomic and proteomic array techniques [33]. Omics is a vernacular expression—the suffix -omics is used frequently to describe something big and refers to a field of study in life sciences that focuses on large-scale data [34]. Proteomics and genomics were the first "omics" formed, and now there are numerous closely related scientific disciplines. Some of these disciplines gave birth to new scientific fields covering narrower arrays of a specific scientific field [35,36].

Biomarkers are actively sought for diseases that slow down society in developed countries (e.g., dementia, renal and cardiovascular disease, and most malignancies). Unfortunately, all the studies in this regard have involved very small numbers of patients and similar numbers of control subjects [37,38]. Finding appropriately matched controls is a true challenge that decreases the odds of clinical validation. This is most often the case when aortic wall tissue is used in proteomics. Opportunities for obtaining a normal-aged aorta as a control are limited, and it can be very daunting. Even if such tissue is obtained, the method and timing of harvest and preservation will modify its protein expression.

3.2. Circulating Markers of the Extracellular Matrix—Biomarkers Related to the Vascular Wall

Collagen fragmentation is typically found in abdominal aortic aneurysm (AAA) biopsies as an indicator of new type I and III collagen synthesis [39]. AAA is rather interesting in the context of postmortem since it bears the risk of rupture or dissection—a life-threatening condition with high mortality [40,41]. The mortality rate is about 25% at 6 hours and 50% by 24 hours, rising to 40%–70% in cases of sepsis [42,43]. Therefore, highly sensitive and specific biomarkers for any vascular pathology should be sought urgently.

Both the carboxy-terminal and amino-terminal ends of the precursor molecule are released during collagen synthesis, and fragments represent candidate biomarkers. Some larger studies and confirmation of clinical validity in a larger cohort are needed to link these molecules to AAA. In this regard, another candidate biomarker was suggested, tenascin-X, due to its implication in Ehlers–Danlos syndrome. AAA patients showed an elevated serum level compared

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to controls [44,45]. Considering that serum elastin peptide (SEP) is a degradation product of elastin, its role as a biomarker has been shifted from sepsis to the extracellular matrix in vascular quiescence, as well [46,47].

Furthermore, examination of the wall of aortic aneurysms has demonstrated medial arterial destruction, accumulation of inflammatory cells, fragmentation of elastin, increased concentrations of proteolytic cytokines, and in situ thrombus [48]. Thus, some additional enzymes, proteins, and cytokines have been explored in this respect. This approach has most often been limited by the fact that all these features represent the end stage of AAA development and may not be indicative of factors initiating AAA development or stimulating AAA growth.

Fragmentation of the extracellular matrix implies elastases and matrix metalloproteinases (MMPs) in the pathophysiology of AAAs. As AAAs are the milieu for abundant expression of MMP-9, this is considered to play a pivotal role in their formation. Thus, this enzyme was explored as a possible biomarker for AAA presence in case–control studies. Patients with AAA demonstrated an elevated circulating MMP-9 concentration [49]. The possible use of elastases as serum biomarkers of extracellular matrix remodeling is considered in the background of the studies involving alpha-1 antitrypsin or p-elastase [50–52]. However, the short half-life of active MMP-9 implies that any active MMP-9 in the serum may have a more immediate origin, so this information could be relevant to clinical forensic scientists [53].

Higher MMP-9 levels are associated with plaque vulnerability in carotid artery atherosclerosis [54]. This is the result of interactions between modified lipids, extracellular matrix, macrophages, and activated vascular smooth muscle cells (VSMCs). Inflammation, lipid accumulation, apoptosis, thrombosis, angiogenesis, and proteolysis all take part in the evolution of atherosclerotic lesions, as these processes are linked to the morphological characteristics of an unstable plaque. The search for a biomarker has focused on these processes [55]. The interplay of vascular wall remodeling and carotid pathology was first hinted at by Makita et al., who linked CRP levels with carotid intima–media complex thickness and plaque formation [56]. Today, there is a known link between obesity in children/adolescents and MMP-9 [52,57]. On the other side, decreases in MMP-3 and MMP-9 have been reported after successful endovascular repair [58,59]. However, these data have extremely limited significance postmortem.

3.3. Proteins Associated with Vascular Lumen—Inflammation and Thrombosis Biomarkers

Inflammation and thrombosis markers are either a final product or an outgrowth of the signaling pathway of a noxious event. Markers of inflammation in vascular disease include cell adhesion molecules, cytokines, pro-atherogenic enzymes, and CRP [52,64]. Biomarkers to identify thrombosis are unlikely to translate into a universal clinical tool; conversely, besides C-reactive protein (CRP), the erythrocyte sedimentation rate (ESR) and procalcitonin (PCT) are widely used [65]. Even hyperhomocysteinaemia has been identified as an indicator of oxidant stress and a significant cardiovascular risk factor [66,67]. However, this association is weak.

The principal markers that have been evaluated are fibrinogen, D-Dimer, homocysteine, and CRP, the elevation of which is intimately linked to other inflammatory cytokines including interleukins (IL-6) and macrophage activation [68,69]. Assessing protein complexes embedded in the coagulation cascade and CRP levels, which are elevated in large aneurysms, covers both processes as a whole [70]. CRP levels decrease quickly, with a half-life of about 19 hours [71].

Out of all acute-phase proteins, CRP is the most commonly investigated biomarker in vascular pathology. Its specific role is to activate the complement cascade in cell death [72], and it is inseparably linked to other inflammatory cytokines [69]. One such cytokine is IL-6, which was confirmed to be a product of AAA [73]. It has its place even in uncomplicated thoracic aortic aneurysms, since the C-reactive protein/interleukin-6 ratio may be a marker of aneurysm size [74]. Also, plasma IL-6 has been correlated to aortic diameter in patients without AAA [17].

Combined with CRP, PCT was challenged as a biomarker for sepsis [75]. In terms of the diagnostic accuracy of CRP for sepsis, the overall area under the summary receiver operator characteristic (SROC) curve was 0.73 (95% confidence interval (CI), 0.69-0.77), with a sensitivity and specificity of 0.80 (95% CI, 0.63-0.90) and 0.61 (95% CI, 0.50-0.72), respectively, and the DOR was 6.89 (95% CI, 3.86-12.31). In terms of the diagnostic accuracy of PCT for sepsis, the overall area under the SROC curve was 0.85 (95% CI, 0.82-0.88), with a sensitivity and specificity of 0.80 (95% CI, 0.69-0.87) and 0.77 (95% CI, 0.60-0.88), respectively, and the DOR was 12.50 (95% CI, 3.65-42.80) [76,77].

The molecular basis of blood coagulation first came to the spotlight in vascular biomarker search when the plasma fibrinogen concentration was found to be positively correlated to the AAA diameter [78]. Nonetheless, its raised plasma concentrations are induced by smoking—the association may only be used as “a black box” of smoking [79]. Due to various functional interactions, fibrinogen plays a crucial role in hemostasis. Specifically, it is a substrate for three major enzymes: thrombin, plasmin, and factor XIIIa [78].

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As clotting slows down, ultimately, the clot breaks down and, together with the fibrin net, dissolves. When they dissolve, fragments of protein are released into the bloodstream. One such specific fragment formed only upon degradation of cross-linked fibrin is d-dimer [80]. Plasma concentrations of D-Dimer show fibrin turnover in the circulation and are, ultimately, related to subsequent mortality from any cause [81]. Most importantly, the D-Dimer level is a routinely used and validated indicator in general clinical practice to exclude a diagnosis of deep vein thrombosis (DVT) [82]. The current serum levels of D-dimer are directly proportional to recent fibrinolytic activity, as the half-life of D-dimer is 4-6 hours. Therefore, measurement of postmortem D-dimer may lead to a limited practical improvement in the current postmortem healthcare.

D-dimer assays available at the time of writing are not standardized, and it is unclear whether these differences have an impact. However, these tests are rapid, simple, and inexpensive [83]. Therefore, to explore differences between D-dimer assays and their impact on the diagnostic outcome, a prospective, multicenter, cohort outcome study evaluating 3462 patients with suspected PE (YEARS study) was considered. Four different D-dimer assays were applied, and the median D-dimer concentrations differed significantly between assays. The sensitivity, specificity, PPV, and NPV for detection of PE of all four assays were determined, using a cutoff level of 1000 ng/mL [7]. In postmortem blood, an Immunochromatographic SERATEC PMB test was used [84]. This test targets human hemoglobin and D-dimer simultaneously, so it is used in forensic inquests for menstrual and peripheral blood spatters [85].

CRP and D-dimer are of utmost interest as they are widely used in clinical work [86]. While the role of both of these molecules as candidate biomarkers in clinical work has been explored, their use in postmortem processing is more a matter of the pathologist's

3.4. Vascular Cognitive Impairment—Room for Postmortem Biomarkers

Vascular cognitive impairment (VCI) is a construct used to capture the entire spectrum of cognitive disorders of the mental abilities to be aware, to think, and to feel. It is associated with a variety of cerebral vascular brain injuries. VCI symptoms can range from forgetfulness to more serious problems with attention, memory, language, and executive functions such as problem solving. Cerebrovascular disease (CeVD) and neurodegenerative dementia such as Alzheimer's disease (AD) are frequently associated comorbidities in the elderly, with the risk factors and pathophysiological mechanisms being essentially alike, including neuroinflammation [87].

Given that it is an inflammatory marker upregulated in vascular diseases and in AD, a protein secreted to plasma—osteopontin (OPN)—has been challenged as a biomarker of AD and VCI [88,89]. Its involvement in lipid metabolism surely explains OPN's role in conditions which fall under the spectrum of VCI. However, among its numerous functions, OPN has emerged as an important potential biomarker for diagnosing and monitoring the treatment of cancer (including melanoma, breast, lung, gastric, and ovarian cancers) and other conditions [90,91].

As information potentially relevant to the practitioner, based on neutralizing OPN with various therapeutic antibody modalities, it is possible to conclude that the half-life of OPN differs depending on the antibody ligand interactions, pH, or "sweeper" used. The calculated half-lives for these four proteins range from 5 to 15 hours [92].

4. Options for Traditional Autopsy

It might be considered distasteful and broadly opposed, but autopsy is an important tool for both criminal investigations and healthcare quality control. For this reason, minimally invasive alternatives to traditional autopsies constantly emerge. Imaging and "verbal autopsy" seem promising when compared with a full autopsy in a large series [28,93–95]. Various objective factors influence the autopsy rate, though it is less likely to be requested for deaths in the emergency department or in general surgery services and most likely to be requested for fetal, medicine, cardiothoracic surgery, and pediatric deaths [96]. Nevertheless, most countries globally do not report autopsy rates (less than 70% of all-cause mortality) [97].

While the cost of electronic data systems and the amount of time between data collection and analysis appear to be the leading disadvantages of verbal autopsies, postmortem imaging is hampered by a lack of direct visualization of soft tissue, as well as postmortem artifacts that obscure natural causes of death and can be misinterpreted as antemortem pathology [95,98].

For deaths occurring outside the health system, health information and a description of events before death are included in a verbal autopsy (VA). A gathering of information about the symptoms and circumstances of death is used to determine the cause of death [99,100]. Scoping the relevant body of knowledge, specificity was commonly found to be higher than sensitivity. Additionally, the negative predictive value was higher than the positive predictive one [101].

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VA is a non-invasive technique where a trained interviewer uses a questionnaire to interview the caregivers of the deceased. Due to its non-contact nature, the World Health Organization's (WHO's) declaration of coronavirus disease 2019 (COVID-19) as a pandemic was an opportunity to explore this autopsy technique [102,103]. It was first used in a public health project concerning the relationships between nutrition, infection, and child development in India [104]. Nowadays, this method is improved and augmented such that it yields an adequately completed death certificate and ultimately estimates cause-specific mortality.

Another accessible and contemporary piece of content from the toolbox is postmortem computed tomography (PMCT). In cases where it has a primary ancillary role in the postmortem examination, it directs forensic pathologists to specific toxicological screening tests [105,106]. Aside from the fact that various imaging techniques can be considered relatively reliable in the medicolegal setting when the patient must be identified, forensic pathologists can benefit from this tool even when in need of guidance during autopsy, fluid analysis, and DNA sampling [107,108]. Most societies treat PMCT imaging as accessible, reproducible, reliable, and easy to implement. Unlike traditional autopsies (TAs), which are invasive procedures, the necessity to fully open the body to perform an autopsy is kept to a minimum with PMCT [109,110].

When assessing the expenses of VA in rural India, the total cost per death was USD 16.66 [111]. The annual cost for the whole population included in the study in year 1 was USD 24,943, inclusive of training. The average annual cost to run the system each year was USD 18,104, and the cost per death was USD 12 for the next 3 years. Costs were reduced by using single physician reviews and shortened re-training sessions.

The explicit potential economic benefits of PMCT (MR) have not been challenged for years [28,29], and in spite of its numerous advantages, this methodology is still burdened with a large ratio of diagnostic discrepancies [112,113]. Nevertheless, PMCT has 79% sensitivity and 92.1% specificity for the detection of the source of bleeding [114]. In another study, where magnetic resonance (MR) and ultrasound (US) were used as imaging modalities, no significant difference in the rates of concordance was reported [115].

A study examining the diagnostic accuracy of postmortem imaging claims that the mean cost of TA was 70% more expensive, so, in the end, having this modality available would leave more financial assets to the institution performing the autopsy [116].

Applying Clinical Biomarkers in a Postmortem Setting

Applying clinical biomarkers in a postmortem setting does not infringe on the medicolegal scrutiny of death investigations. Instead of limiting the contents of the death investigation toolbox, it could be used to decrease discrepancies in clinical autopsy and to reduce postmortem healthcare inequalities [21,22,117].

At the time of this review, only few countries have published data for both the autopsy rate and gross national product (GNP), so the correlation between the number of autopsies and GNP is weak and negative (r^2=-0.38; p=0.004). Subject discrepancies were minimized across time and then increased significantly in the last few years [118,119]. In a further installment of research, no significant difference was found in treatment time between hemorrhagic and ischemic lesions seen later during brain autopsy (unpublished data [120]).

Predominately, as a consequence of the decline in clinical (hospital) autopsy, overall autopsy rates have declined during the past decades in many high-income countries [121]. This negative trend has been attributed to various factors such as costs, lack of medical education, development of new clinical diagnostic tools, medical malpractice implications, and difficulties in obtaining permission from relatives [122]. Even when performed, some autopsies tend to be negative—without any findings that reveal the cause of death. On the other hand, studies show substantial discrepancies between autopsy results and pre-mortal clinical diagnoses [123,124]. This is most clearly visible in the global autopsy rates in all-cause mortality as a part of the World Health Organization's (WHO's) annual statistics [125,126]. Even Paratz et al. reported these rates varying within the range 0.01%–83.9%, based on the data from the less than one-third of countries that report autopsy rates at all [97]. Their statistics are mostly derived from academic periodicals rather than governmental data.

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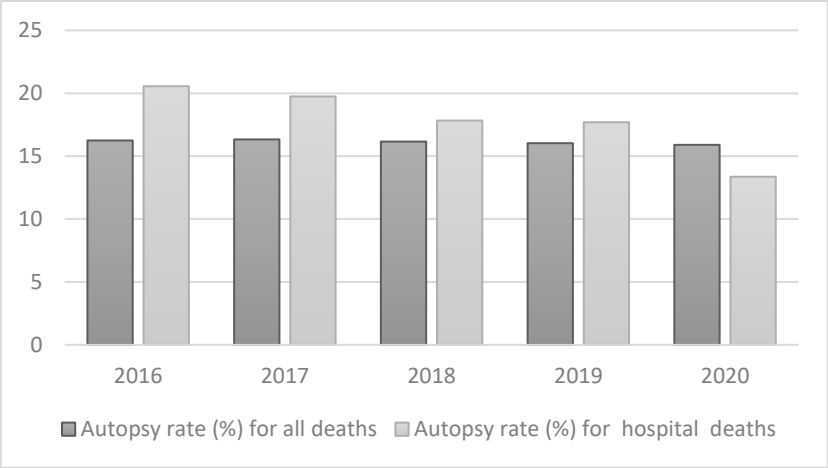

Figure 1. Five-year autopsy rate for all-cause mortality worldwide, 2016–2020. A slight decline in the overall autopsy rate can be seen, along with a more evident decrease in the autopsy rate for hospital deaths (more than 4% for the year 2020 compared to 2019).

While healthcare practices have come a long way in reducing mortality, decreasing the number of TAs requires the availability of a feasible alternative. Nonetheless, any form of postmortem investigative tool can provide additional information or a change of diagnosis regarding the cause of death in a great number of cases, either through discrepancies between clinical and autopsy diagnosis, or through inconclusive autopsies. In order to maintain the viability of academic departments included in postmortem care and to increase consent for postmortem investigations, a panel of noninvasive biomarkers is proposed, given in Table 1.

Table 1. Possible blood-based biomarkers of vascular disease.

Related Process	Biomarker	Medium	Reference values
Inflammation	**C-reactive protein (CRP)**	S	< 0.3 mg/dL: Normal (level seen in most healthy adults); 0.3 to 1.0 mg/dL: Normal or minor elevation (can be seen in obesity, pregnancy, diabetes, common cold, gingivitis, periodontitis, sedentary lifestyle, cigarette smoking, and genetic polymorphisms) [60]
	Osteopontin (OPT)	S	122.3 ± 39.2 ng/mL
		P	463.7 ng/mL - 587.0 ng/mL [61]
Related to thrombus	**D-Dimer**	S	<2,152 ng/mL [62]
Matrix-degrading enzymes	**MMP-9**	S	mean 436 ng/mL (range, 169-705 ng/mL) [63]

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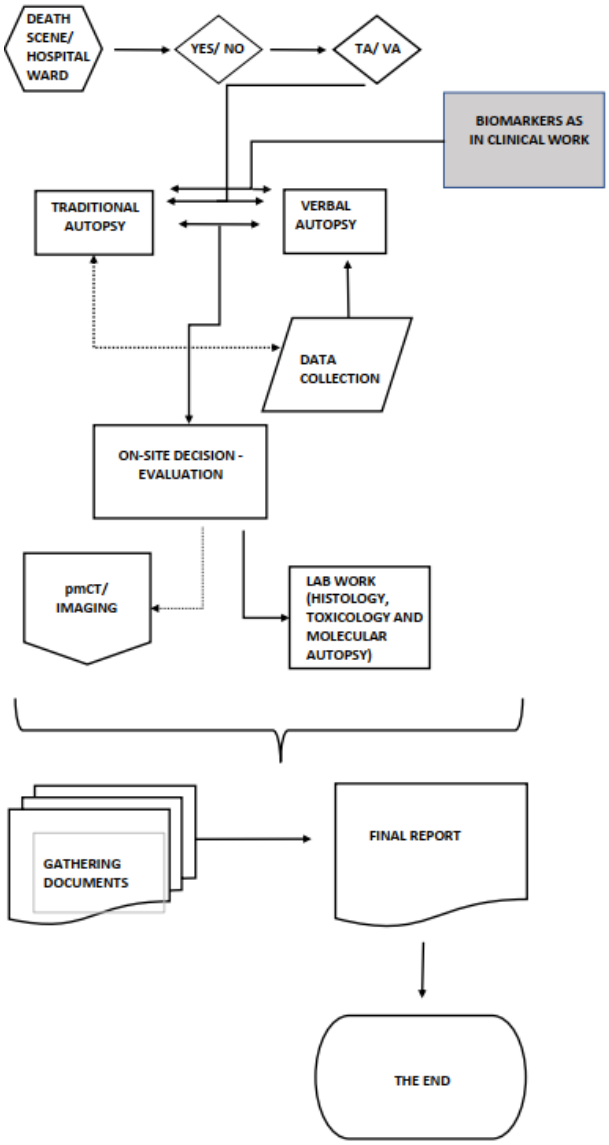

Figure 2. Flowchart of the tentative postmortem protocol with biomarkers included.

The aim for this panel of clinical biomarkers is to improve these statistics and to influence the large discrepancy between clinical diagnosis and autopsy findings. This discrepancy ranged from 7,2% in a study by Stambouly et al. in 1993 to 64% in a study by Mitrovic et al. in 2019 [127,128].

5. Conclusions

Autopsies are still needed for the control and correction of causes of death, even in "clear-cut" cases. Postmortem sample handling and analysis remain the challenges in the variability of the findings, and this is central in the validation of biomarkers. These limitations do exist because no published large-scale study has considered postmortem human blood samples. Risks of bias include the inability to verify reported figures, heterogeneity in the reporting of clinical vs. medicolegal autopsies, and the limited number of studies concerning the vascular pathology overall.

Considering the half-lives of all the candidate molecules that are in the framework of this review, it is not very likely for any of these molecules to have wider applicability. However, each one of the highlighted markers could prove useful in confirming or ruling out a cause of death in cases of witnessed deaths or in situations where TA is not an option. In conclusion, further work is needed in search of new candidate molecules.

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